

Production of pilot lots of inactivated influenza vaccines from reassortants derived from avian influenza viruses

Interim biosafety risk assessment





Production of pilot lots of inactivated influenza vaccines from reassortant derived from avian influenza viruses

Interim biosafety risk assessment



WORLD HEALTH ORGANIZATION
DEPARTMENT OF COMMUNICABLE DISEASE
SURVEILLANCE AND RESPONSE

© World Health Organization 2003

All rights reserved.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

The World Health Organization does not warrant that the information contained in this publication is complete and correct and shall not be liable for any damages incurred as a result of its use.

Contents

1	Introduction.....	2
2	Scope of the risk assessment	2
3	Virus rescue	3
4	Pathogenicity testing	3
5	Vaccine production	3
6	Hazard identification	3
6.1	Hazards associated with the recipient virus	3
6.2	Hazards arising from the inserted gene product	3
6.3	Hazards arising from the alteration of existing pathogenic traits.....	3
6.4	Potential hazards of sequences being transferred to related micro-organisms	6
6.5	Likelihood of harm to human health	6
7	Assignment of a provisional containment level.....	7
8	Nature of the work and review of control measures to safeguard human health	7
9	Environmental hazards and any additional control measures necessary	7
	References	9
	Annex 1 Testing for attenuation of influenza vaccine strains in mammals .	11
	Test virus.....	11
	Laboratory facility	11
	Ferrets	11
	Mice.....	12
	Annex 2 Participating partners	14

1 Introduction

The 1997 and 2003 cases of human H5N1 infections in Hong Kong and the 2003 cases of human H7N7 infections in the Netherlands were caused by highly pathogenic avian influenza viruses. It is generally accepted that continued human exposure to influenza viruses circulating in wild and domestic avian species poses a pandemic threat. Worldwide efforts, in addition to veterinary measures and farm practices, are now under way to develop emergency prophylactic measures for pandemic influenza. These measures include the rapid development of safe vaccine strains capable of growth in eggs or mammalian cells as soon as a pandemic warning is issued, and the production of vaccine according to epidemiological demands.

The influenza virus genome consists of 8 segments. It is likely that a high growth reassortant will provide the basis for pandemic vaccine development, although it is conceivable that an apathogenic wild-type avian virus could be used. Should a reassortant be used, it is likely to contain the haemagglutinin (HA) and neuraminidase (NA) proteins from the novel avian or human virus and the remaining 6 proteins derived from a human influenza virus, such as A/PR/8/34 (H1N1). If a suitable apathogenic virus is available, reassortants may be produced by conventional technology. In the event that no suitable apathogenic strain is available, the vaccine virus will be a reassortant containing the HA of a highly pathogenic influenza virus, where the HA gene has been modified to remove the multi-basic amino acids at the HA connecting peptide. The modifications to the HA will be done to remove the known determinants of high pathogenicity.

Reassortants will be produced by reverse genetics using either an 8 plasmid or 12 plasmid strategy.

8 plasmid system

Eight plasmids, each encoding one of the influenza virus genes will be under the direction of the pol I-pol II expression system.

12 plasmid system

Eight plasmids, each encoding one of the influenza virus genes will be under the direction of the pol I expression system. Four plasmids will express PB1, PB2, PA and NP proteins.

2 Scope of the risk assessment

This document was developed by WHO Influenza Collaborating Centers for Research and Reference on Influenza, National Influenza Centers and other partners - the participating list is attached as Annex II. The draft document was sent to the whole influenza society in the world, including more than 1600 influenza experts and other colleagues from academies, national licensing agencies, pharmaceutical industries and research institutions interested in influenza surveillance and control, for comments. We received concrete comments from more than 40 individuals, institutions and pharmaceutical companies. All comments received were reviewed and integrated into the final document.

The generation of reassortants derived from highly pathogenic avian influenza viruses is carried out under BSL3+ or 4 conditions. After demonstration of non pathogenicity, reassortants are available to vaccine manufacturers for production of pilot lots of vaccine for experimental use and clinical studies. This interim risk assessment is intended to provide guidance to vaccine manufacturers on biosafety measures, but each manufacturer should prepare its own risk assessment, taking into account local working practices, local biosafety control measures and

local environmental control measures. The examples used in this risk assessment relate to an H5N1 vaccine development project, but the arguments are applicable to vaccine production from any potential pandemic virus. As more information becomes available, the risk assessment will be updated and should the need arise for large scale vaccine production, a revised risk assessment will be produced.

3 Virus rescue

Virus will be rescued from plasmid transfection of a Vero cell line approved for human vaccine production. Reassortant virus will contain six internal gene protein segments of PR8 virus and the NA and modified HA segment of the avian virus. The virus will subsequently be grown in eggs or in mammalian cells.

4 Pathogenicity testing

Virus pathogenicity will be assessed in chickens and ferrets (Annex 1). Mice may also be used, depending on the pathogenicity of the avian virus for mice.

5 Vaccine production

Each company to briefly describe its own production process.

6 Hazard identification

6.1 Hazards associated with the recipient virus

The recipient influenza virus will be the human strain PR8. This is a virus that has had extensive passage in mice, ferrets and chicken eggs. The result of such a passage history is almost complete inability to replicate in man and complete attenuation for man (Beare et al., 1975).

The reason for selecting PR8 is its capacity for high growth in not only mammalian cells, but also in embryonated chicken eggs. Since the late 1960s, PR8 virus has been used to produce "high growth reassortants" in combination with the prevailing influenza A vaccine strain and use of such reassortants as vaccine strains has increased vaccine yield many-fold. These reassortants have been produced by a combination of mixed infection of eggs with PR8 and the vaccine strain and a selection system based on use of anti-PR8 antibody and growth at high dilution.

There are no known risks to human health from the PR8 virus.

6.2 Hazards arising from the inserted gene product

The products of the inserted genes will be the modified HA and NA of an H5N1 virus. The HA will have been modified so that the multi-basic amino acids at the HA connecting peptide will be reduced to a single basic amino acid. These influenza proteins alone are not inherently infectious or harmful.

6.3 Hazards arising from the alteration of existing pathogenic traits

The influenza HA spike protein has specificity for sialic acid receptors on cell surface molecules. The HA's present on human influenza A viruses preferentially bind to cell receptors containing α

2, 6 linked sialic acid residues, whereas avian influenza viruses preferentially bind to α 2, 3 linked sialic acid (Rogers and D'Souza, 1989). Human tracheal cells have mainly α 2, 6 linked residues (Nelson et al, 1993), so the acquisition of an avian HA by PR8 virus is expected to minimize potential binding to human respiratory epithelial cells.

Although the α 2, 3 receptor specificity of avian viruses will reduce the efficacy of such binding, it may not completely prevent infection in man. Beare and Webster (1991) were successful in infecting volunteers with a variety of avian influenza viruses, although replication was poor. However Beare and Webster found that extremely large quantities of avian viruses (between $10^{6.8}$ and $10^{9.2}$ egg infectious doses) were needed for replication in man and that it was not possible to induce person-to-person transmission.

In 1997, infections of man with an avian H5N1 virus also took place in Hong Kong, although replication in these cases was much better, probably due to the virulent nature of the virus. In Hong Kong, people were exposed to high titre H5N1 virus in contaminated faeces, which may have been one of the reasons for virus transmission from birds to man. However, as in the experimental studies, there was little or no person-to-person transmission of the 1997 H5N1 virus. This was also the case with the more limited occurrence of human infection with the avian H9N2 virus in 1999. Therefore in conclusion, the presence of an H5 HA on the surface of the H5N1xPR8 reassortant virus is likely to exhibit extremely weak binding to human cells with low probability of human infection.

The HA protein of influenza virus must be cleaved into HA1 and HA2 by host cell proteases for a productive infection. Pathogenicity of H5 and H7 influenza A viruses for poultry is largely determined by the presence of multi-basic amino acids at the HA connecting peptide. The HA's of highly pathogenic viruses can be effectively cleaved by ubiquitous furin-like proteases, which are expressed in most organs of birds and man. However, the HA's of non-pathogenic viruses contain a single basic residue at the connecting peptide which can only be cleaved by trypsin-like proteases which are restricted to certain cell types, e.g. epithelial cells lining the respiratory tract of man and the gut of birds. Thus HA cleavability determines tissue specificity and is a major determinant of pathogenicity.

Direct evidence has been obtained that both HA cleavage and HA receptor specificity have an effect on tissue tropism of an avian H7N1 virus, A/Fowl Plague/Rostock/34 in chicken embryos (Feldmann et al., 2000). Similarly, the available evidence from the 1997 H5N1 infections demonstrates that the high degree of pathogenicity in chickens, mice and ferrets was directly related to the possession of multi-basic amino acids. Studies performed at the WHO CC Tokyo (M Tashiro, unpublished data) have demonstrated that removal of the basic amino acids changed H5N1 infections from a fatal systemic infection to a localized non-pathogenic infection in chickens, mice and ferrets. Hatta et al. (2001) have also shown by reverse genetics that high cleavability of H5N1 HA due to the presence of multi-basic amino acids, was an essential requirement for a lethal mouse infection.

While it is not possible to examine pathogenicity of influenza virus infection in man, an examination of H5N1 viruses by Gao et al. (1999) has provided evidence that pathogenicity in mice resembles that in man. The occurrence of multiple organ failure after human H5N1 infections is suggestive of an unusual tissue tropism, but no evidence for viral replication outside the lung has been found (To et al., 2001). The available evidence therefore suggests that virulence of the 1997 H5N1 viruses for man was related to the presence of multi-basic amino acids. For these reasons, removal of the basic amino acids from the 2003 H5N1 virus HA is considered advisable in order to reduce the potential for harm to man. This procedure will also increase safety of the reassortants for avian species. Further information is provided in the environmental risk assessment section below.

The choice of the PR8 strain for reassortment is also based on its proven attenuation for man. Published information indicates that a PR8 reassortant with a 6:2 genotype (6 segments from PR8, HA and NA from a wild-type human influenza virus) is avirulent in man (Florent, 1980; Beare and Hall, 1971; Beare et al., 1975; Oxford et al, 1978). Indeed, Florent et al. (1977) and studies performed at the WHO CC Tokyo (M Tashiro, unpublished data) have shown that the degree of attenuation increases as reassortants include more PR8 genes. In this project, reassortants derived from the 2003 H5N1 virus will contain six out of eight viral genes from PR8, which is the maximum achievable within the scientific aims of the work. It is therefore envisaged that a reassortant bearing 6 internal protein genes of PR8 virus and the NA and modified HA of the H5N1 virus will also be attenuated for man.

All the above evidence on virus replication in man is based on reassortants with HA's derived from human influenza viruses, which preferentially bind to cell receptors abundant in human respiratory epithelium (α 2, 6 linked sialic acid residues). The reassortants created in this project contain an avian H5 HA which has a preference for α 2, 3 linked residues, so that there is little expectation that the H5N1 reassortants will be able to bind to and replicate in human cells.

While it is clear from the Hong Kong experience of 1997 that H5N1 influenza viruses, which displayed α 2, 3 sialic acid specificity, could replicate in humans, it must be noted that influenza virus pathogenicity does not depend solely on HA, but is a polygenic trait, and that the 1997 H5N1 virus had unusual PB2 and NS1 genes which influenced pathogenicity. Changes in the PB2 gene of the 1997 H5N1 viruses were sufficient to attenuate them for mice (Hatta et al., 2001) and changes in the NS1 protein rendered these viruses resistant to the effects of interferons and other cytokines produced as part of the innate immune response (Seo et al., 2002). The NS1 changes conferred a highly virulent phenotype which allowed replication to proceed unchecked in vivo.

In this case even a virus with a poor affinity for its receptor was able to replicate (although not to transmit). In contrast viruses with a PR8 internal protein gene constellation were clearly sensitive to the innate immune restrictions which prevent the establishment of infection by an avian virus in humans. This may well explain why in avian influenza outbreaks before 1997, no evidence of transmission from birds to man has been noted. This may also explain why, during many years of laboratory handling of high titre avian viruses (one of which [A/FPV/Dobson] is known to contain a gene which adapts it for replication in mammalian cells), no workers have apparently been affected by these viruses (Almond, 1977).

The H5N1xPR8 reassortants created in this project will not contain the gene constellation considered necessary for pathogenicity in chickens, mice and ferrets.

Reassortants derived from PR8 have been used routinely for production of inactivated influenza vaccines for the past 30 years. This work involves production of many thousands of litres of infected egg allantoic fluids, which will create substantial aerosols of reassortant virus within manufacturing plants. Most of the reassortants were made from wild-type human strains that had not yet been in widespread circulation. Thus, although the manufacturing staff would have some susceptibility to infection with the wild-type virus, there have been no anecdotal or documented cases of work-related human illness resulting from exposure to the reassortants. This is further testimony to the attenuation of PR8 reassortants. Nevertheless, unlike the situation with normal vaccine production, manufacturing staff for pilot lots of a potential pandemic vaccine would have no previous immunological experience of an avian virus, so staff would be expected to be susceptible, although the risk is expected to be low.

Genetic stability of reassortant viruses is an important issue as the wild-type non-pathogenic H5 and H7 avian viruses are the source of highly pathogenic viruses. Studies of a non-pathogenic H5N3 reassortant between A/Goose/Hong Kong/437/99 and PR8 have shown no evidence of

reversion to virulence (chickens, mice and ferrets) after 10 passages in eggs (R Webster, unpublished data).

The reassortant H5N1 viruses will be assessed and found negative for pathogenicity in the statutory chicken intravenous pathogenicity test (IVP index of 1.2 or less) (OIE, 2001) and in ferrets (virus replication and clinical symptoms consistent with those induced by the attenuated parent virus [eg PR8] and distinguishable from the H5N1 avian virus infection) (Annex 1). Tests for safety in mice may also be performed. The reassortant virus may then be distributed to vaccine manufacturers.

Ferrets have extensively been used as a good indicator of influenza virus virulence for man (reviewed by Smith and Sweet, 1988). Typically, human influenza viruses cause lethargy, nasal discharge and occasionally fever; virus replication is usually limited to the respiratory system. PR8 virus has been assessed in ferrets; it causes little or no clinical symptoms and virus replication is limited to the upper respiratory tract. However the 1997 Hong Kong H5N1 virus replicated throughout the body, caused fever, weight loss and occasionally death (Zitzow et al., 2002). Thus in terms of predicting a highly pathogenic human infection or an infection which is attenuated for man, the ferret is the best available model.

6.4 Potential hazards of sequences being transferred to related micro-organisms

Influenza viruses readily exchange genes by the process of reassortment. Thus there is a theoretical possibility that secondary reassortants could occur between the newly created H5N1 x PR8 reassortant and naturally occurring human or animal influenza viruses. Although it is considered that the H5N1 x PR8 reassortant will be non-infectious and attenuated for man, a secondary reassortant with a human influenza virus may be infectious for man and pose an epidemic threat.

It is generally considered to be technically difficult to produce reassortants *in vitro* and only a few laboratories in the world have success with this technique. Moreover the chance of producing reassortants between mammalian and avian viruses is extremely limited, as was demonstrated by the lack of success in producing H5N1 reassortant vaccine in 1997 (UK, avian and swine viruses; Australia and USA, avian and PR8 viruses). When such difficulties are considered, together with the unlikely event that the laboratory containment measures would allow an H5N1 x PR8 virus to infect man and produce a secondary reassortant, the risk of such an event is low.

It should also be considered that poultry and pig farmers are continually exposed to animal influenza viruses and there have been few documented cases of human infection with a reassortant between an avian and human influenza virus. The risk of such secondary reassortments for animal species will be considered in the environmental risk assessment section.

6.5 Likelihood of harm to human health

By virtue of avian receptor specificity, PR8 attenuation and loss of multi-basic amino acids at the HA connecting peptide, it is envisaged that the H5N1 x PR8 reassortant will not be capable of infecting man or causing harm to human health. As described above, there is a remote possibility of secondary reassortment with normal human viruses and such reassortants may be replication-competent in man, although avian receptor barriers would still act to restrict infection. In an extreme situation, such a reassortant could become well adapted to human infection and cause epidemic activity around the world.

However, the likelihood of such an event is low.

7 Assignment of a provisional containment level

The parental PR8 virus is a hazard group 2 biological agent and the HA of the H5N1 virus will be engineered so that the rescued virus will be non-pathogenic.

In view of the low likelihood of harm to human health, the provisional containment level will be biosafety level 2+ (BSL2 with additional controls in place, i.e. BSL2+)

8 Nature of the work and review of control measures to safeguard human health

Each laboratory must review its own control measures in light of the intended work and the nature of the laboratory facilities, however the following may be used as a guide:

- Ideally a BSL2+ laboratory should be maintained at an air pressure negative to the atmosphere and all virus manipulations outside sealed containers should take place within a microbiological safety cabinet. However this may not be possible in a manufacturing environment and alternative control measures therefore be needed:
 - use of other suitable barrier systems;
 - where virus manipulations on the open bench are unavoidable, staff should be protected by use of powered full-face respirators, equipped with HEPA filters;
 - antiviral prophylaxis for staff in the production area and those in adjacent areas.
- Showering is not required, as protective clothing and hand washing procedures are normally considered adequate to protect human health and the environment for this level of hazard.
- There should be no need to inactivate effluent from hand basins and sinks, because any liquid effluent from sinks should have been disinfected by validated procedures and there is little risk of hand-washing effluent posing a hazard to the environment.

A code of practice for the work should be prepared, the key features of which are:

- Procedures to prevent exposure of the H5N1 reassortant to normal human and animal influenza viruses. Staff should have received a conventional influenza vaccine to limit their susceptibility to infection with normal human viruses. If pilot lots of H5 vaccine are available, staff should receive them. There should also be an Occupational Health Policy for antiviral prophylaxis or for treatment following accidental exposure to the H5N1 reassortant virus.
- Review of all working practices to minimize the creation of aerosols from the vaccine virus.
- Procedures for the safe decontamination of waste and equipment.
- Emergency procedures for events such as spillages documented.
- Staff training programme documented.

9 Environmental hazards and any additional control measures necessary

Influenza viruses are capable of naturally infecting a variety of animal species (birds, pigs, horses, man, aquatic mammals, ferrets) although there are host restrictions which limit the host range of certain virus subtypes. As the H5N1 reassortant will have avian receptor specificity, birds would be the species theoretically most susceptible.

What would be the contribution of PR8 internal genes to replication and virulence in birds? Brown et al. (2001) demonstrated that adaptation of an influenza H3N2 virus to increased virulence in mice could result in a variety of mutations in different virus genes. Three H3N2 mutations were in common with the virulent Hong Kong H5N1 virus and one (PA – 556) was shared with PR8 virus. Thus it could be argued that acquisition of PR8 genes may indicate increased risk for animals.

However Hatta et al. (2002) have recently demonstrated, by the use of reverse genetics, that acquisition of only one PR8 gene by an avian influenza virus abrogates virus replication in ducks. Based on this work, an avian virus with six internal protein genes of PR8 virus would not be expected to replicate in birds. Indeed, experimental evidence has demonstrated that PR8 virus is attenuated in not only man (already discussed) but also chickens (Subbarao et al., 2003). Furthermore a reassortant between PR8 (internal protein genes) and the 1997 Hong Kong H5N1 virus (NA and HA with single basic amino acid) was barely able to replicate in chickens and was not lethal. Similar studies have been performed with the 2003 Hong Kong H5N1 virus at WHO CC Memphis (R Webster, unpublished data), where the PR8 reassortant did not replicate or cause disease signs in chickens. The removal of the multi-basic amino acids from the H5 x PR8 reassortants in both studies probably played a role in reducing the risk for chickens.

It is conceivable that pigs are susceptible to infection by the H5N1 reassortant, as viruses with avian receptor specificity are known to replicate in this species. It is also possible that these species would be susceptible to secondary reassortments between the H5N1 reassortant and a pig virus. There is in fact evidence that triple reassortants between avian, pig and human influenza viruses can circulate in pigs in the USA (Webby et al., 2000).

Each laboratory to assess the risk of avian or porcine infection based on the likelihood of avian species or pigs being in the vicinity and the laboratory controls in use.

The laboratory code of practice for this work prevents work taking place with other animal influenza viruses at the time of the reassortant work, thus eliminating the risk of additional reassortment events within the laboratory. It is also known that mice can be experimentally infected with some influenza viruses: the PR8 strain is known to be lethal for mice and the H5N1 reassortant is able to replicate in mice. Steps should therefore be taken to prevent exposure of wild mice and escape of laboratory mice.

Each laboratory to comment on the rodent control measures in place

Therefore in summary, there are no additional measures needed to protect the environment.

Assignment of containment level

BSL2+

References

Almond JW (1977). A single gene determines the host range of influenza virus. *Nature*, 270:617-618.

Beare AS, Hall TS (1971). Recombinant influenza A viruses as live vaccines for man. *Lancet*, 2:1271-1273.

Beare AS, Schild GC, Craig JW. (1975). Trials in man with live recombinants made from A/PR/8/34 (H0N1) and wild H3N2 influenza viruses. *Lancet*, 2:729-732.

Beare AS, Webster RG (1991). Replication of avian influenza viruses in humans. *Archives of Virology*, 119:37-42.

Brown EG et al. (2001). Patterns of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. *Proceedings of the National Academy of Sciences of the United States of America*, 98: 6883-6888.

Couceiro JNSS et al. (1993). Influenza virus strains selectively recognize sialoligosaccharides on human respiratory epithelium: the role of the host cell in selection of haemagglutinin receptor specificity. *Virus Research*, 29:155-165.

Feldmann A et al. (2000). Targeted infection of endothelial cells by avian influenza virus A/FPV/Rostock/34 (H7N1) in chicken embryos. *Journal of Virology*, 74:8018-8027.

Florent G (1980). Gene constellation of live influenza A vaccines. *Archives of Virology*, 64:171-173.

Florent G et al. (1977). RNAs of influenza virus recombinants derived from parents of known virulence for man. *Archives of Virology*, 54:19-28.

Gao P et al. (1999). Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *Journal of Virology*, 73:3184-3189.

Hatta M et al. (2001). Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science*, 293:1840-1842.

Hatta M et al. (2002). Human influenza A viral genes responsible for the restriction of its replication in duck intestine. *Virology*, 295:250-255.

Katz JM et al. (2000). Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *Journal of Virology*, 74:10807-10810.

Lu X et al. (2003). Pathogenicity and antigenicity of a new influenza A (H5N1) virus isolated from duck meat. *Journal of Medical Virology*, 69:553-559.

Matsuyama T et al. (1980). Aspects of virulence in ferrets exhibited by influenza virus recombinants of known genetic constitution. *Journal of Infectious Diseases*, 141:351-361.

OIE (2001). Manual of Standards for Diagnostic Tests and Vaccines. 4th Edition. Office International des Epizooties, 12 rue de Prony, 75017 Paris, France. www.oie.int.

Oxford JS et al. (1978). Analysis of virion RNA segments and polypeptides of influenza A virus recombinants of defined virulence. *Nature*, 273:778-779.

Reed LJ, Muench H (1938) . A simple method for estimating fifty percent endpoints. *American Journal of Hygiene*, 27:493-497.

Reuman PD, Keely S, Schiff GM (1989). Assessment of signs of influenza illness in the ferret model. *Laboratory Animal Science*, 42:222-232.

Rogers GN, D'Souza BL (1989). Receptor binding properties of human and animal H1 influenza virus isolates. *Virology* , 173:317-322.

Seo SH, Hoffman E, Webster RG (2002). Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nature Medicine*, 9:950-954.

Smith H, Sweet C (1988). Lessons for human influenza from pathogenicity studies with ferrets. *Reviews of Infectious Diseases*, 10:56-72.

Subbarao K et al. (2003). Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology*, 305:192-200.

To K et al. (2001). Pathology of fatal human infection associated with avian influenza A H5N1 virus. *Journal of Medical Virology*, 63:242-246.

Webby RJ et al. (2000). Evolution of swine H3N2 influenza viruses in the United States. *Journal of Virology*, 74:8243-8251.

Zitzow LA et al. (2002). Pathogenesis of avian influenza A (H5N1) viruses in ferrets. *Journal of Virology*, 76:4420-4429.

Annex 1 Testing for attenuation of influenza vaccine strains in mammals

The recovered vaccine candidate containing six internal gene segments of PR8 and the NA and modified HA of the avian virus will be assessed for their ability to cause disease in ferrets following intranasal infection. Studies in mice may also be considered as additional data on virus attenuation. For optimal interpretation of results, the pathogenic properties of the vaccine strain should be compared, ideally in the same experiment, with the parental PR8 and wild-type avian viruses. The following are guidelines for the experimental procedure and assessment of expected outcomes.

Test virus

The 50% infectious dose of an egg- or mammalian cell-passaged stock of vaccine virus and the parental viruses will be determined by titration in eggs (EID₅₀) or cells (TCID₅₀) as appropriate. Titration of vaccine virus stock and parental virus stocks should be determined within the same laboratory and titres should be sufficiently high such that these viruses can be compared using equivalent high doses in mice or ferrets (10^7 to 10^6 EID₅₀ or TCID₅₀). Ideally, different laboratories will use a common PR8 donor strain, since passage history can alter the virulence for mice. The virulence properties of the donor PR8 should be characterized thoroughly in each laboratory.

Laboratory facility

Animal studies with the vaccine strain and the parental wild-type H5N1 strain should be conducted in biosafety level 3+ (BSL3+) containment facilities. Investigators should wear personal air-powered respirators (PAPRs) and an appropriate occupational health policy should be in place.

Ferrets

Background: The H5N1 viruses isolated from humans in Hong Kong in 1997 caused severe disease in ferrets. The viruses caused substantial weight loss, fever, and severe lethargy and resulted in occasional neurologic sequelae and/or death. Isolation of virus from systemic organs on days 1-5 post-infection and neuropathological findings on day 14 were also associated with the enhanced virulence in ferrets (Zitzow et al, 2002). However, another highly pathogenic H5N1 virus isolated from duck meat imported into Korea from China in 2001 exhibited none of these properties and was apparently apathogenic in ferrets (Lu et al, 2003). Thus, the wild-type H5N1 parental strain, along with the PR8 donor of internal genes, must be carefully evaluated for virulence in ferrets. Studies by others have indicated that PR8 is not virulent and replicates poorly in ferret lungs (Matsuyama et al, 1980), although these properties should be confirmed for the actual donor strain used in vaccine strain preparation. While it would be ideal to conduct the pathogenicity testing of the vaccine strain and the parental strains simultaneously, this may not be feasible in most laboratories due to space limitations. In this case, careful evaluation of the parental strains prior to evaluation of the vaccine strain is recommended for each laboratory conducting tests.

Experimental procedure: Outbred ferrets 4-8 months of age are sedated either by intramuscular inoculation of a mixture of anaesthetics (e.g. ketamine [25 mg/kg], xylazine [2 mg/kg] and atropine [0.05 mg/kg]) or by a suitable inhalant. A standard dose of 10^7 EID₅₀/TCID₅₀ (as appropriate) (10^6 , if the higher dose is not possible) in 1 ml phosphate-buffered saline is slowly distilled onto the nares of the sedated animal, making sure that the virus is inhaled and not

swallowed or expelled. A group of 4-6 ferrets should be infected. One group of ferrets (2-3 animals) will be euthanized on day 3 or 4 post-infection and the following tissues should be collected for estimation of virus replication: nasal turbinates and/or swabs, lung (tissue samples from each of 4 lobes and pooled), brain (tissues from anterior and posterior sections sampled and pooled), spleen or intestine. Additional lung tissue may be collected and processed for hematoxylin and eosin (H&E) staining for microscopic evaluation of histopathology. The remaining animals are observed for 14 days for signs of weight loss, lethargy (based on a previously published index [Reuman, 1989]), respiratory and neurological symptoms. Neurological involvement may be confirmed by collection of brain tissue on day 14 post-infection at the termination of the experiment and processing as above for histopathology.

Expected outcome: Viral titres of the vaccine strain in respiratory tissues should be no greater than either parental strain; a substantial decrease in lung virus replication is anticipated. Replication of the vaccine candidate should also be restricted to the respiratory tract and replication in the spleen or intestine is not expected. While isolation of the vaccine strain from the brain is not desirable, if high viral titres are found in the nasal turbinates there may be some detection of virus in the brain based on previous results with non-virulent human H3N2 viruses (Zitzow, 2002). The significance of such a finding may be confirmed by performing a histopathological analysis of brain tissue on day 14 p.i. Neurological lesions detected in H&E stained tissue sections should confirm virus replication in the brain and observation of neurological symptoms. Neurological symptoms and histopathology would indicate a lack of suitable attenuation of the vaccine candidate. Likewise clinical signs of disease such as weight loss and lethargy should indicate lack of attenuation in the vaccine strain, assuming that the wild-type avian virus also causes these symptoms.

Mice

Background: Highly pathogenic H5N1 viruses isolated in Asia since 1997 are highly infectious for BALB/c mice and require no adaptation to this host. Although these viruses all replicate to high titres in the respiratory tract of mice, they differ in their ability to disseminate systemically, replicate in the brain and cause lethal disease. Viruses may be broadly classified into two groups: those that have low lethality for mice ($LD_{50} > 10^{6.0} EID_{50}$) and those that are highly lethal for mice ($LD_{50} < 10^{3.0} EID_{50}$). Lethality and ability to replicate outside the respiratory tract have been associated with the presence of a multi-basic amino acid cleavage site in the HA. This cleavage site appears to be necessary but not solely responsible for extrapulmonary spread of the virus and high lethality. A substitution in PB2 (627 Glu→Lys) is associated with high lethality of the 1997 H5N1 viruses in mice, although other as yet undefined substitutions also contribute to this property (Hatta et al, 2001; Katz et al, 2000).

Since not all highly pathogenic avian strains are highly virulent (lethal) in mice, the main utility of the mouse as a test system for reassortant safety will be in cases where the parental avian strain is inherently virulent for mice. When avian strains that are not virulent for mice are used, reassortment with the PR8 virus may confer virulence on the vaccine reassortant strain since PR8 itself is virulent for mice.

Experimental procedure: The 50% lethal dose (LD_{50}) of the vaccine strain and parental virus strains is determined in 6-8 week old female BALB/c mice. Mice are lightly anesthetized with an inhalant and groups of mice (4-8 per group) are infected intranasally with 0.05 ml of serial 10-fold dilutions of virus (expected dose range 10^7 to $10^1 EID_{50}$). Mice are observed daily for disease signs and the numbers of deaths at each virus dilution are recorded. The LD_{50} values are calculated by the method of Reed and Muench (1938). An additional 3 mice infected with a high dose of virus (e.g. 10^6) are sacrificed on day 3 or 4 post-infection and organs, including the lungs and brain, are harvested for estimation of virus replication.

Expected outcome: If the wild-type avian strain replicates in the brain and is highly lethal for mice, the vaccine candidate should exhibit at least a 1000-fold reduction in lethality (i.e. ≥ 3 log increase in the LD₅₀ value). Lung and brain titers of the vaccine strain should be lower than those of either parental strain, consistent with an attenuation of replication in mouse tissues.

Annex 2 Participating partners

1. WHO GIP: WHO Global Influenza Programme, Department for Communicable Disease Surveillance and Response, Avenue Appia, CH-1211 Geneva 27, Switzerland
2. WHO CC Atlanta: WHO Collaborating Centre for Influenza Surveillance, Epidemiology and Control, Influenza Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, 1660 Clifton Rd NE, Atlanta, GA 30333, United States of America
3. WHO CC London: WHO Collaborating Centre for Reference and Research on Influenza, Research Division of Virology, National Institute of Medical Research, The Ridgeway, Mill Hill, London NW7 1 AA, United Kingdom
4. WHO CC Melbourne: WHO Collaborating Centre for Reference and Research on Influenza, 45 Poplar Road, Parkville, Victoria 3052, Australia
5. WHO CC Tokyo: WHO Collaborating Centre for Reference and Research on Influenza, Laboratory of Respiratory Viruses, National Institute of Health, 1-23-1 Toyama, Shinjuku Ku, Tokyo 162, Japan
6. WHO CC Memphis: WHO Collaborating Centre for Influenza, Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, 332 N Lauderdale, Memphis, TN 38105, United States of America
7. CBER, Bethesda: Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, Rockville, MD 20892, United States of America
8. NIBSC Potters Bar: National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom
9. TGA Woden: Therapeutics Goods Administration Laboratories, PO Box 100, Woden ACT, Australia
10. HK University: Department of Microbiology, University of Hong Kong, University Pathology Building, Queen Mary Hospital, Pokfulam Road, Hong Kong SAR
11. NIC H K: Government Virus Unit, Public Health Laboratory Centre, 382 Nam Cheong Street, Shek Kip Mei, Kowloon, Hong Kong SAR